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Roles of viral and cellular proteins in the expression of alternatively spliced HTLV-1 pX mRNAs[1]

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Abstract

The human T cell leukemia virus type 1 (HTLV-1) genome contains a cluster of at least five open reading frames (ORFs) near the 3′ terminus within the pX region. The pX ORFs are encoded by mono- or bicistronic mRNAs that are generated by alternative splicing. The various pX mRNAs result from skipping of the internal exon (2-exon versus 3-exon isofoms) or from the utilization of alternative splice acceptor sites in the terminal exon. The Rex and Tax proteins, encoded by ORFs X-III and X-IV, have been studied intensively and are encoded by the most abundant of the alternative 3-exon mRNAs. The protein products of the other pX ORFs have not been detected in HTLV-1-infected cell lines and the levels of the corresponding mRNAs have not been accurately established. We have used real-time RT-PCR with splice-site specific primers to accurately measure the levels of individual pX mRNA species in chronically infected T cell lines. We have asked whether virus regulatory proteins or ectopic expression of cellular factors influence pX mRNA splicing in cells that were transfected with HTLV-1 provirus clones. In chronically infected cell lines, the pX-tax/rex mRNA was present at 500- to 2500-fold higher levels than the pX-tax-orfII mRNA and at approximately 1000-fold higher levels than pX-rex-orfI mRNA. Chronically infected cell lines that contain numerous defective proviruses expressed 2-exon forms of pX mRNAs at significantly higher levels compared to cell lines that contain a single full-length provirus. Cells transfected with provirus expression plasmids expressed similar relative amounts of 3-exon pX mRNAs but lower levels of 2-exon mRNA forms compared to cells containing a single, full-length provirus. The pX mRNA expression patterns were nearly identical in cells transfected with wild-type, Tax-minus, or Rex-minus proviruses. Cotransfection of cells with HTLV-1 provirus in combination with SF2/ASF expression plasmid resulted in a relative increase in pX-tax/rex mRNA compared to pX-tax-orfII and pX-rex-orfI mRNAs, but did not affect exon skipping. Ectopic expression of hnRNP A1 did not affect pX splice site utilization, but increased exon skipping, as the level of pX-p21rex mRNA was increased by almost 10-fold.

Introduction

Approximately 20 million people worldwide are infected with human T cell leukemia virus type 1 (HTLV-1), a retrovirus that establishes a persistent and lifelong infection. A subset of infected individuals develop either adult T cell leukemia (ATL) or a neurodegenerative disease referred to as tropical spastic paraparesis or HTLV-associated myelopathy (TSP/HAM), usually decades after the initial infection (Bangham, 2000; Johnson et al., 2001; Manns et al., 1999). Although other cells can be infected with HTLV-1 in vivo and in vitro, the primary cellular target of the virus is the T-lymphocyte (Cho et al., 1995; Hanon et al., 2000; Koyanagi et al., 1993; Nagai et al., 2001; Richardson et al., 1990).

The HTLV-1 genome contains a cluster of at least five open reading frames (ORFs) located near the 3′ end that are highly conserved among HTLV-1 isolates. These pX region ORFs encode proteins that have known or putative *trans*-regulatory functions (Fig. 1A). The Rex and Tax proteins, encoded by pX ORF X-III and ORF X-IV, respectively, are

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essential for virus expression and replication. Tax interacts with a variety of cellular proteins to activate transcription of the virus promoter, to redirect the cellular gene expression program, and to alter cell-cycle control (Ressler et al., 1996; Yoshida, 1993, 2001). Rex mediates the nuclear-cytoplasmic transport of intron-containing viral mRNAs (Johnson et al., 2001).

The functions of pX ORFs X-I, X-II, and X-V have been more difficult to unravel, since the predicted proteins have not been detected in HTLV-I-infected cell lines and are not essential for virus replication in vitro (Collins et al., 1999; Derse et al., 1997). However, in vivo expression of ORF X-I and ORF X-II is supported by the observation that infected individuals and experimentally infected animals developed humoral and cell-mediated immune responses to the respective peptides (Dekaban et al., 2000; Pique et al., 2000). Furthermore, HTLV-1 mutants defective for expression of ORFs X-I and X-II were unable to establish persistent infections in experimentally infected animals (Bartoe et al., 2000; Collins et al., 1998). Ectopic expression of recombinant X-I and X-II ORFs have provided some biochemical clues relevant to their biological functions. Ectopic expression of the 12-kDa, C-terminal portion of ORF-XI ($p12^{I}$) revealed that it accumulated in the endoplasmic reticulum and Golgi apparatus (Koralnik et al., 1993; Ding et al., 2001). Biochemical studies showed that $p12^{I}$ was associated with vacuolar H^{+} ATPase (Franchini et al., 1993), MHC I heavy chain (Johnson et al., 2001), IL-2 receptor β and γ chains (Koralnik et al., 1995, Mulloy et al., 1996), and calreticulin (Kim et al., 2003). Although the effects of $p12^{I}$ on T cell activation pathways have been reported (Albrecht et al., 2000, 2002; Collins et al., 1999; Nicot et al., 2001; Ding et al., 2002), there is no consensus as to its essential biological functions. Cells transfected with an ORF X-II expression plasmid produced a 30-kDa nuclear protein ($p30^{II}$) that was reported to inhibit the expression of CREB-responsive promoters (Zhang et al., 2000, 2001). Expression of ORF X-V has not been carefully evaluated since the first HTLV-I isolate to be completely sequenced had a stop codon in this reading frame (Seiki et al., 1983); however, ORF X-V is uninterrupted in the majority of HTLV-1 provirus sequences available in the GenBank database.

HTLV-1 structural and regulatory proteins are expressed from differentially spliced mRNAs (Fig. 1A). The unspliced mRNA encodes *gag*, *pro*, and *pol* polyproteins. Splicing from exon 1 in the 5′ LTR to an exon 2 splice acceptor site in the middle of the viral genome generates a singly spliced mRNA encoding the envelope glycoprotein. Further splicing from exon 2 to splice acceptor sites in the pX region yields a set of multiply spliced mRNAs. The most abundant and best studied of these mRNAs is generated using a splice acceptor site at position 6950 (positions are numbered with respect to virus mRNA) that encodes Tax and Rex. Alternative pX splice acceptor sites at positions 6383, 6478, and 6875 were defined by cloning RT-PCR products derived from infected cell lines and patient samples (Berneman et al., 1992; Caputo and Haseltine, 1992; Cereseto et al., 1997; Ciminale et al., 1992; Koralnik et al., 1992). Translation of the alternatively spliced pX mRNAs is predicted to follow the pattern established for Tax and Rex. These proteins are translated from a bicistronic mRNA using two different initiation codons in exon 2 such that 20 amino acids from exon 2 are joined to the Rex ORF or one residue (the other initiator methionine) is joined to the Tax ORF (Fig. 1B). In addition to the mRNAs that contain exon 2, cDNA clones have been isolated in which exon 1 was joined directly to the pX splice acceptors. The mRNA in which exon 1 is spliced to the *tax/rex* acceptor at position 6950 was previously shown to produce a truncated version of Rex (p21Rex), which initiates at an internal methionine codon (Shuh et al., 1999). Likewise, shortened forms of ORFs X-I and X-II are predicted to originate by translation initiation at internal AUG codons (Fig. 1B).

The pX proteins, other than Tax and Rex, have not been detected in HTLV-1-infected cell lines, and the relative levels of individual, alternatively spliced pX mRNAs have not been accurately established. Previous methods lacked the sensitivity and selectivity necessary to resolve a complex mixture of closely related RNAs. We employed real-time RT-PCR with splice site-specific primers to quantify the levels of individual pX mRNAs. In chronically infected T cell lines, pX mRNAs encoding ORFs X-I, X-II, and X-V were present at levels that were two to four orders of magnitude lower than the tax/rex mRNA. We also examined pX mRNA expression profiles in cells transfected with wild-type and mutant HTLV-1 provirus clones to ask whether Tax or Rex proteins influenced pX mRNA expression patterns. Finally, we asked whether overexpression of the cellular splicing factors SF2/ASF or hnRNP A1 could modulate alternative splicing of HTLV-1 pre-mRNA.

Results

HTLV-1 RNA splicing

The splicing pattern for HTLV-1 is depicted in Fig. 1A. Exon 1 is bounded by the mRNA start site at position 1 and the major splice donor site at position 119. Exon 2 is defined by alternative splice acceptor sites at positions 4641 and 4658 and by the splice donor site at position 4831. Utilization of alternative splice acceptor sites at positions 6383, 6478, 6875, or 6950 in the pX region generates a nested set of four terminal exons. Alternatively spliced pX mRNAs can be divided into two groups: mRNAs of the first group contain exon 1, exon 2, and one of the four terminal pX exons, while mRNAs in the second group are composed of exon 1 joined directly to one of the four pX exons. Translation of the mRNAs that include exon 2 is best understood for the bicistronic pX-tax/rex mRNA, which uses the pX, splice acceptor at position 6950 (Fig. 1B). By analogy, the mRNAs in which exon 2 is spliced to the acceptor sites at

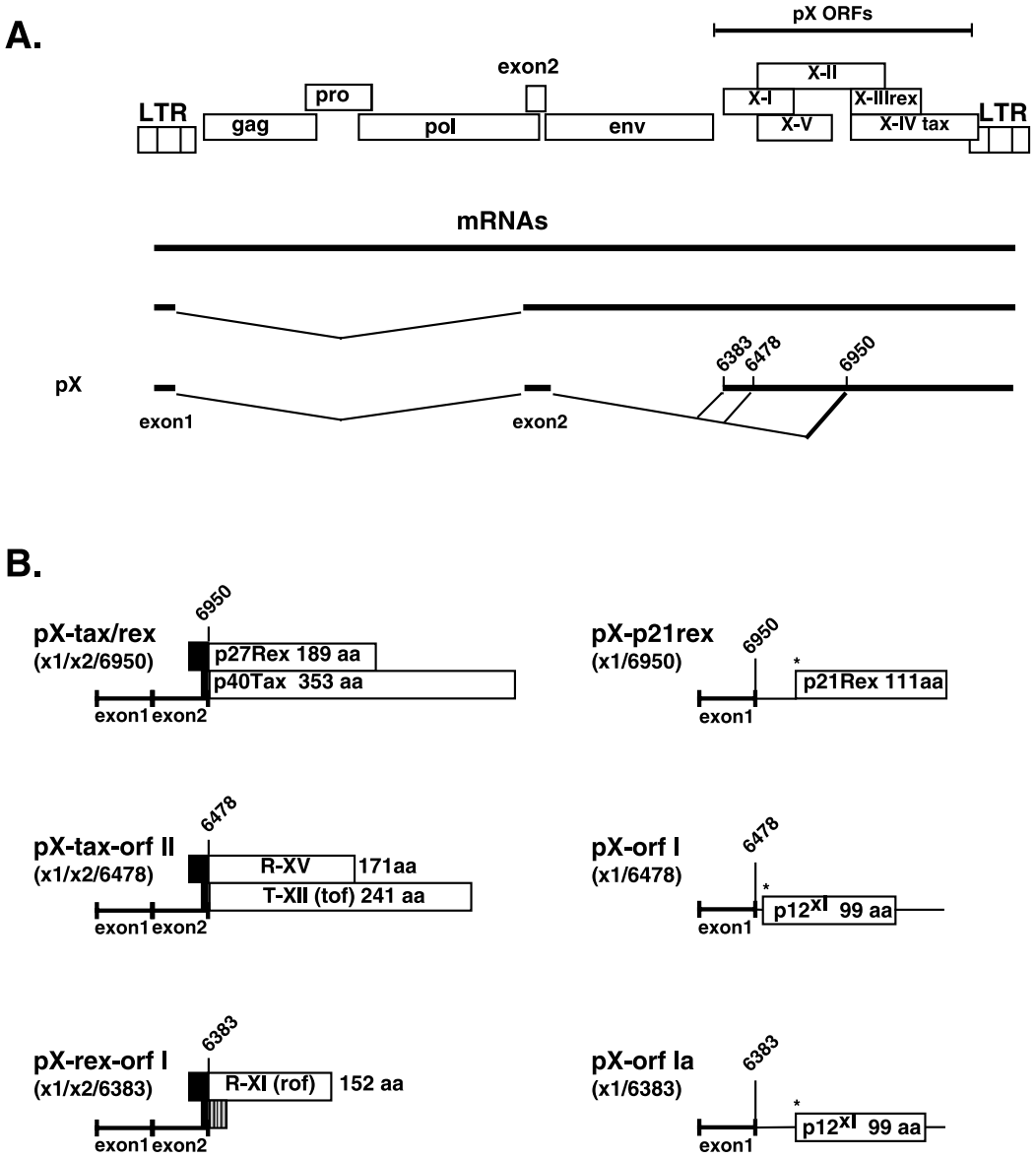
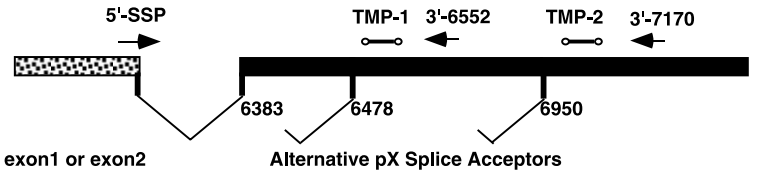


Fig. 1. HTLV-1 genetic organization, mRNAs, and known or predicted pX gene products. (A) The 3′ end of the HTLV-1 genome encodes five open reading frames collectively referred to as the pX region. The Rex and Tax proteins are encoded by ORFs X-III and X-IV, respectively, in the distal half of pX. Products of ORFs X-I, X-II, and X-V, located in the proximal half of pX, have not been detected in HTLV-1-infected cells. The various HTLV-1 mRNAs are produced by alternative splicing of the genomic mRNA, which encodes *gag*, *pro*, and *pol* genes. The singly spliced mRNA encodes *env* and multiply spliced mRNAs encode the various pX proteins, including Tax and Rex. The pX splice acceptor sites examined here are designated by their positions in viral mRNA and include 6383, 6478, and 6950. Asterisks above exon 2 of the pX mRNAs designate translation start codons for Rex (upstream) and Tax (downstream). Not shown are the pX mRNAs that exclude exon 2; i.e., that splice from exon 1 directly to one of the pX exons. (B) The known or predicted translation products derived from pX mRNAs that include exon 2 (left) or exclude exon 2 (right) are shown. The mRNAs are designated according to the earlier convention based on predicted translation products and by the exons present and the pX splice acceptor site in the terminal exon, in parentheses. In addition, mRNAs are designated, in parentheses. Translation of the mRNAs that include exon 2 are predicted to start at the Rex (R) or Tax (T) initiation codons. Translation of the mRNAs that exclude exon 2 are assumed to start at the first in-frame initiation codon (asterisk). Predicted sizes are given in amino acids (aa).

positions 6383 and 6478 are predicted to yield proteins containing ORFs X-I, X-II, and X-V. The mRNAs that lack exon 2 are predicted to initiate translation at internal AUG codons within the pX ORFs as was previously demonstrated for the mRNA containing exon 1 joined to the acceptor site at 6950, yielding pX-p21^{rex} mRNA. Since proteins derived from ORFs X-I, X-II, and X-V have not been detected in HTLV-1-infected cells, we focused on quantifying the steady-state levels of the mRNAs for these proteins. The set of six pX mRNAs that include or exclude exon 2 and that use splice acceptors at positions 6383, 6478, and 6950 were examined. The pX mRNAs are designated by their deduced translation products or by the exon content and position of the pX splice acceptor site in the terminal exon; e.g., pX-



mRNA	Junction	5'-Splice-Specific Primer	3' Primer	Taqman Probe
pX-rex-orf I	(ex2/6383)	ACCAACACCATGG^ CAACT	3'-6552	TMP-1
pX-tax-orf II	(ex2/6478)	ACCAACACCATGG^ CACTA	3'-6552	TMP-1
pX-tax/rex	(ex2/6950)	ACCAACACCATGG^ CCCA	3'-7170	TMP-2
pX-orf Ia	(ex1/6383)	GTCCGCCGTCTAG^ CAAC	3'-6552	TMP-1
pX-orf I	(ex1/6478)	GTCCGCCGTCTAG^ CACT	3'-6552	TMP-1
pX-p21rex	(ex1/6950)	GTCCGCCGTCTAG^ CCCA	3'-7170	TMP-2

Fig. 2. Splice site-specific PCR strategy for real-time PCR amplification of HTLV-1 mRNAs. The pX mRNAs are generated by alternative splicing of either exon 1 or exon 2 to one of the pX splice acceptor sites at positions 6383, 6478, or 6950. The 5′ splice site-specific primers are complementary to 13 nucleotides in exon 1 or exon 2 with four or five nucleotides at the 3′ end of the primer complementary to a specific pX exon. The splice junction is designated in parentheses next to each mRNA and indicated in the primer sequences by (^). PCR amplification specificity is conferred by nucleotide differences at the 3′ end of the 5′-splice-specific primer, by the location of the 3′ primers (3′-6552 and 3′-7170), and by the Taqman probes (TMP-1 or TMP-2).

tax/rex mRNA is ×1/×2/6950 (Fig. 1). An additional pX mRNA was previously identified that used a splice acceptor at position 6875 and is predicted to encode $p13^{II}$; however, we did not examine its expression here.

Real-time RT-PCR conditions

To solve the problem of specifically quantifying an individual cDNA in a complex mixture of closely related species, we employed splice site-specific 5′ (sense) primers paired with appropriate 3′ (antisense) primers and Taqman probes (Fig. 2). The 5′ portions of the splice site-specific primers are complementary to approximately 13 bases in the upstream exon and the 3′ portion of the primer is complementary to four or five bases in the respective downstream exon. For each alternatively spliced pX mRNA a corresponding cDNA clone was generated and this plasmid DNA was used to establish optimal real-time PCR reaction conditions, to calibrate cDNA copy number, and to determine primer specificity.

Real-time PCR reactions were performed with serial 10-fold dilutions of the appropriate cDNA plasmid to give amounts ranging from 2.5 to 2.5×10^7 copies per reaction. Threshold cycle values (Ct) values, defined as the cycle at which PCR products were detected above the designated threshold level, were plotted against the $\log_{10}$ of the plasmid copy number. Calibration plots for all primer pairs used in this study were linear between 10 and 10^7 copies of plasmid DNA targets with correlation coefficients of at least 0.97 and slopes of approximately −3.4.

To determine the specificity of each set of splice site-specific primers, we compared real-time PCR amplification kinetics with 10^6 copies of either cognate or noncognate cDNA plasmid templates. For each primer pair, the Ct value obtained with its cognate cDNA plasmid was between 16.4 and 17.7. The Ct values for noncognate plasmids were at least 10 cycles, and generally 20 cycles higher compared to cognate cDNAs (data not shown). Primer specificity may be expressed as the difference between the Ct values for cognate and noncognate cDNAs with a difference between Ct values of approximately 3 equal to a 10-fold difference in cDNA copy number. Thus, with respect to cDNA concentrations, specificity for a cognate cDNA was three to six orders of magnitude greater than for an alternative cDNA. This overall specificity is further demonstrated by the results obtained with cDNAs from HTLV-1-infected cell lines shown below.

pX mRNA levels in chronically infected cell lines

The HTLV-1-infected T cell lines chosen for analysis have been characterized with respect to provirus copy number and provirus sequence. The MS9 cell line was established by infection of primary human T cells with the molecular clone, pHTLV-X1MT, and contains a single, full-length provirus (Shuh et al., 1999). MT2 cells contain

Table 1

cDNA	cDNA copy number		Relative copy number	
	MS9	MT2	MS9	MT2
pX-tax/rex ×1/×2/6950	$1.93 \pm 0.84 \times 10^6$	$1.35 \pm 0.19 \times 10^5$	1.0×10^6	1.0×10^6
pX-tax-orf II ×1/×2/6478	$7.72 \pm 4.85 \times 10^2$	$2.75 \pm 0.58 \times 10^2$	4.0×10^2	2.0×10^3
pX-rex-orf I ×1/×2/6383	$2.90 \pm 0.40 \times 10^3$	$1.84 \pm 0.48 \times 10^2$	1.5×10^3	1.4×10^3
pX-p21rex ×1/6950	$1.28 \pm 0.16 \times 10^5$	$1.87 \pm 0.12 \times 10^6$	6.6×10^4	1.4×10^7
pX-orfI ×1/6478	$6.30 \pm 2.25 \times 10^3$	$9.75 \pm 2.85 \times 10^3$	3.3×10^3	7.2×10^4
pX-orfIa ×1/6383	$1.30 \pm 0.52 \times 10^3$	$7.82 \pm 5.52 \times 10^2$	6.7×10^2	5.8×10^3

Note. pX mRNA levels in chronically infected cell lines. HTLV-1 pX cDNA copy numbers were determined as described under Materials and Methods and are normalized relative to 1.0×10^6 copies of GAPDH cDNA. Mean values from at least three independent experiments are shown with standard deviations. Relative copy numbers are expressed with respect to 1.0×10^6 copies of pX-tax/rex cDNA.

one full-length and at least seven internally deleted HTLV-1 proviruses (Kobayashi et al., 1984). The latter proviruses have deletions encompassing exon 2 and produce only the exon 1/pX group of spliced mRNAs. Poly(A^+) mRNA was purified from each cell line, converted to cDNA using random hexamer primers, and analyzed by real-time PCR with the splice site-specific primer pairs (Fig. 2).

Copy numbers of alternatively spliced pX mRNAs were calculated from calibration plots using cDNA plasmids then normalized to a GAPDH cDNA copy number of 1.0×10^6 to compare pX mRNA levels between the cell lines (Table 1).

The total pX mRNA copy number was similar in MS9 and MT2 cells. In MS9 cells the most abundant pX mRNA was the 3-exon pX-tax/rex (×1/×2/6950) isoform, which was present at approximately 15-fold higher levels than the 2-exon pX-p21rex (×1/6950) mRNA (Table 1). The relative amounts of these two mRNAs were inverted in MT2 cells compared to MS9 cells. The higher levels of pX-21rex (×1/6950) mRNA in MT2 cells is attributed to the large number of internally deleted proviruses in this cell line. The amounts of pX-tax/rex (×1/×2/6950) and pX-p21rex (×1/6950) mRNAs in the two cell lines were proportional to the amounts of p40Tax, p27Rex, and p21Rex proteins that were previously determined by immunoblotting (Shuh et al., 1999). The level of pX-rex-orf I (×1/×2/6383) mRNA was about 700-fold lower than pX-tax/rex mRNA in both cell lines. The pX-tax-orf II (×1/×2/6478) mRNA was present at 2500-fold lower levels than pX-tax/rex mRNA in MS9 cells and 500-fold lower than pX-tax/rex mRNA in MT2 cells. In MS9 cells, the 2-exon pX-orf I (×1/6478) and pX-orf Ia (×1/6383) mRNAs were present at levels 300- and 1500-fold, respectively, lower than the pX-tax/rex mRNA. In MT2 cells the relative levels of these 2-exon mRNAs were 10- to 20-fold higher than in MS9 cells.

pX mRNA splicing in cells transfected with wild-type and mutant HTLV-1 proviruses

We next examined pX mRNA accumulation in cells transiently transfected with HTLV-1 provirus expression plasmids (Table 2). Levels of the 3-exon pX-tax-orf II (×1/×2/6478) and pX-rex-orf I (×1/×2/6383) mRNAs were 200- and 300-fold lower, respectively, relative to pX-tax/rex mRNA. These values are comparable to those seen in the chronically infected cell lines. However, levels of the 2-exon mRNAs, pX-p21rex (×1/6950), pX-orf Ia (×1/6383), and pX-orf I (×1/6478) were significantly lower in transfected cells compared to either of the chronically infected cell lines. To test the possibility that viral *trans*-regulatory proteins, Tax or Rex, might exert feedback control of pX mRNA splicing, pX mRNA levels were compared in cells transfected with either the wild-type (pCMV-HT1), Tax-minus (pCMVHT-Tax9Q), or Rex-minus (pCMVHT-Rex1L) versions of HTLV-1 proviruses (Table 2). Provirus expression was under the control of the CMV immediate-early promoter to ensure uniform and Tax-independent levels of RNA synthesis. There was no significant difference in the relative distribution of pX mRNAs in cells transfected with wild-type, Tax-minus, or Rex-minus proviruses, indicating that Tax and Rex do not regulate alternative splicing.

Effects of SF2/ASF or hnRNP A1 overexpression on pX mRNA levels

Alternative splicing may be regulated by the cell type dependent or condition-specific activities of splicing factors. Both constitutive and alternative RNA splicing are

Table 2

cDNA	Relative copy number		
	wt	TAX^-	REX^-
pX-tax/rex ×1/×2/6950	1.0×10^6	1.0×10^6	1.0×10^6
pX-tax-orfII ×1/×2/6478	5.00×10^3	5.57×10^3	3.56×10^3
pX-rex-orfI ×1/×2/6383	2.81×10^3	1.15×10^3	2.09×10^3
pX-p21rex ×1/6950	3.62×10^3	3.77×10^3	4.53×10^3
pX-orfI ×1/6478	74	46	68
pX-orfIa ×1/6383	142	41	32

Note. pX mRNA levels in cells transfected with wild-type (wt) and mutant HTLV-1 provirus expression plasmids. HTLV-1 pX cDNA copy numbers were determined as described under Materials and methods and are normalized relative to 1.0×10^6 copies of pX-tax/rex cDNA. Mean values from at least three independent experiments are shown. Standard deviations were within the range shown in Table 1.

controlled by *cis*-acting splicing enhancer or suppressor elements that recruit various cellular accessory proteins (Ladd and Cooper, 2002). Among these, SF2/ASF and hnRNP A1 have been shown to positively or negatively control, respectively, alternative splicing of pre-mRNA (Caceres et al., 1994; Amendt et al., 1994; Bilodeau et al., 2001; Blanchette and Chabot, 1999; Chung and Derse, 2001; Damgaard et al., 2002; Fu, 1995; Gontarek and Derse, 1996; Jacquenet et al., 2001; Sun et al., 1993; Tange et al., 2001; Tange and Kjems, 2001). We asked whether overexpression of either SF2/ASF or hnRNP A1 in cells transfected with the HTLV-1 provirus affects HTLV-1 pX mRNA splicing. To compare the levels of pX mRNAs among the transfected cell conditions, pX mRNA levels were expressed relative to pX-tax/rex (×1/×2/6950) mRNA (Table 3). In cells transfected with pCMV-HT1, overexpression of SF2/ASF resulted in a decrease in the relative levels of pX-tax-orf II (×1/×2/6478) and pX-rex-orf I (×1/×2/6383) mRNAs of 17- and 22-fold, respectively (Table 3). That is, in the group of 3-exon pX mRNAs, SF2/ASF overexpression promoted the utilization of the splice acceptor site at position 6950. Overexpression of SF2/ASF did not significantly alter the relative levels of the 2-exon versus 3-exon mRNAs, indicating that it did not affect exon 2 recognition.

Unlike SF2/ASF, overexpression of hnRNP A1 did not significantly affect pX splice acceptor site utilization among the 3-exon pX mRNAs, since the relative levels of these mRNAs remained unchanged (Table 3). However, cotransfection with the hnRNP A1 expression plasmid induced a 6.5-fold increase in the level of pX-p21rex (×1/6950) mRNA and a 5-fold increase in the relative level of pX-orf Ia (×1/6383) mRNA. A similar effect was not observed for pX-orfI (×1/6478) mRNA, but the levels of this mRNA were at the lower limits of detection. Thus, hnRNP A1 overexpression promoted exon 2 exclusion and increased the levels of the 2-exon pX mRNAs relative to the 3-exon mRNAs.

Table 3

cDNA	Relative copy number		
	Vector control	+SF2/ASF	+hnRNP A1
pX-tax/rex ×1/×2/6950	1.00×10^6	1.00×10^6	1.00×10^6
pX-tax-orfII ×1/×2/6478	5.00×10^3	0.29×10^3	3.06×10^3
pX-rex-orfI ×1/×2/6383	2.81×10^3	0.13×10^3	2.62×10^3
pX-p21rex ×1/6950	3.62×10^3	2.89×10^3	23.5×10^3
pX-orfI ×1/6478	74	29	49
pX-orfIa ×1/6383	142	480	717

Note. pX mRNA levels in cells transfected with HTLV-1 provirus expression plasmid in combination with empty vector (vector control), SF2/ASF, or hnRNP A1 expression plasmids. HTLV-1 pX cDNA copy numbers were determined as described under Materials and methods and are normalized relative to 1.0×10^6 copies of pX-tax/rex cDNA. Mean values from at least three independent experiments are shown. Standard deviations were within the range shown in Table 1.

Discussion

Real-time RT-PCR with splice site-specific primers provided the methodology necessary to accurately quantify each cDNA specific for an individual pX mRNA isoform. The method has a wide dynamic range and circumvents the problem of competition or interference among target molecules inherent in other approaches. We first determined the relative levels of the various pX mRNAs in chronically infected cell lines and then asked whether viral regulatory proteins or cellular splicing factors affected their patterns of expression in transiently transfected cells. The HTLV-1 infected cell lines, MS9 and MT2, were characterized previously with respect to viral protein expression and provirus copy number, genetic organization, and nucleotide sequence. In cell lines such as MS9, which contain a single full-length provirus, or in cells transfected with full-length provirus clones, the pX-tax/rex (×1/×2/6950) mRNA was the most abundant of the pX mRNAs. In MS9 cells the second most abundant pX mRNA was pX-p21rex (×1/6950) mRNA. In MT2 cells, which contain multiple internally deleted proviruses, the relative amounts of these two mRNAs were reversed; pX-p21rex mRNA was 15-fold higher than pX-tax/rex mRNA. Relative levels of pX-tax/rex and pX-p21rex mRNAs in MS9 and MT2 cells determined here were proportional to the amounts of p40Tax, p27Rex, and p21Rex proteins previously determined by immunoblotting (Shuh et al., 1999).

Besides pX-tax/rex mRNA, two other pX mRNAs include exon 2 and thus join the tax or rex translation initiation codons to pX ORFs X-I, X-II, or X-V. pX-tax-orf II (×1/×2/6478) mRNA is predicted to encode a 30-kDa Tax-ORF II protein ($p30^{II}$) and a 21-kDa Rex-ORF V protein. In ectopic expression experiments, $p30^{II}$ was shown to be a nuclear protein (Koralnik et al., 1993) which may have a role in regulating virus gene expression (Zhang et al., 2000, 2001). The pX-rex-orf V gene product has not been studied yet because the first HTLV-1 isolate to be sequenced had a stop codon interrupting this ORF (Seiki et al., 1983); this appears to be the exception, since most other HTLV-1 sequences in the GenBank database predict an extended open reading frame. The pX-tax-orf II mRNA was detected at levels that were lower than pX-tax/rex mRNA by a factor of 2500-fold in MS9 cells, 500-fold in MT2 cells, and 200-fold in provirus-transfected cells. The pX-rex-orf I (×1/×2/6383) mRNA encodes a Rex-ORF-I fusion protein of approximately 19 kDa. Ectopic expression of this cDNA resulted in the detection of a 12-kDa protein, $p12^{I}$, which may be generated by translation initiation at an internal start codon or by posttranslational cleavage of the larger precursor protein (Ciminale et al., 1992; Koralnik et al., 1992, 1993). Biochemical analyses of $p12^{I}$ expressed in transfected cells indicated that it was associated with several different membrane-associated cellular proteins (Franchini et al., 1993; Johnson et al., 2001; Koralnik et al., 1995; Mulloy et al., 1996; Ding et al., 2001; Kim et al., 2003) and

affects T cell signal transduction pathways (Albrecht et al., 2000, 2002; Nicot et al., 2001; Ding, 2002). The pX-rex-orf I mRNA was detected at levels that were 700-fold lower than pX-tax/rex mRNA in MT2 and MS9 cells and 360-fold lower in cells transfected with HTLV-1 provirus. The pX-orf I (×1/6478) and pX-orf Ia (×1/6383) mRNAs do not include exon 2 and therefore would initiate translation at a start codon within ORF X-I to produce $p12^{I}$. In MS9 cells pX-orf I and pX-orf Ia mRNAs were expressed at about 300- and 1500-fold lower levels, respectively, compared to pX-tax/rex mRNA. In MT2 cells, these mRNAs, similar to the pX-p21rex mRNA, accumulated at higher levels than in MS9 cells. In contrast, pX-orf I and pX-orf Ia mRNAs were barely detectable in cells transfected with HTLV-1 provirus. Assuming that relative mRNA levels are proportional to the levels of their encoded proteins, it is not surprising that the ORF X-I and ORF X-II gene products have not been detected in HTLV-1-infected cell lines.

The two previous attempts to estimate the levels of alternatively spliced pX mRNAs in cells transfected with a cloned HTLV-1 provirus or in HTLV-1-infected cell lines gave results quite different than those determined here. In the former case, Northern blotting was used to show that pX-rex-orf I and pX-tax-orf II mRNAs together were expressed at about 20-fold lower levels than the singly spliced env mRNA (Ciminale et al., 1992). Since this method could not distinguish between the two pX mRNAs and because of its narrow dynamic range, Northern blotting provided only an approximate estimation of relative mRNA levels. In another study, RNase protection assays with probes that spanned alternative mRNA splice sites were employed to examine pX mRNA levels in various HTLV-1-infected cell lines (Cereseto et al., 1997). While this method has much greater sensitivity than Northern blotting, there are still inherent problems with specificity and quantification when applied to complex mixtures of closely related mRNAs. These problems are exemplified by results which showed that pX-tax-orf II mRNA was expressed at two- to seven-fold higher levels than the pX-tax/rex mRNA in two HTLV-1-transformed cell lines (Cereseto et al., 1997). These values are not consistent with our quantitative real-time PCR data nor with previous Northern blotting results (Ciminale et al., 1992).

There is strong immunological data indicating that the proteins encoded by ORF X-I and ORF X-II mRNAs, $p12^{I}$ and $p30^{II}$, are expressed in infected individuals and in experimentally infected animals (Dekaban et al., 2000; Pique and Dokhelar, 2000; Pique et al., 2000). Furthermore, experimental infections with cloned proviruses indicated that $p12^{I}$ and $p30^{II}$ were necessary for virus propagation and persistence in vivo (Bartoe et al., 2000; Collins et al., 1998). In contrast, these proteins have not been detected, and their mRNAs are expressed at very low levels, in chronically infected cell lines; in addition, ORF X-I and X-II genes are dispensable for HTLV-1 infection or transformation of T cells in vitro (Collins et al., 1999; Derse et al., 1997). The expression of pX ORFs of HTLV-1 is reminiscent of the papillomavirus oncogenes. Papillomaviruses are small DNA tumor viruses that establish persistent infections of epithelial cells and induce epithelial hyperplasias that occasionally progress to malignant neoplasms (Stubenrauch and Laimins, 1999). In addition to a transcriptional activator (E2), bovine papilloma virus (BPV-1) encodes three regulatory proteins (E5, E6, and E7) that alter the cellular environment and have oncogenic activity. Expression of these three proteins is dependent on cell differentiation and repressed by viral regulatory proteins E1 and E2 (Vande Pol and Howley, 1995). The BPV-1 E5 protein was observed in fibropapillomas; E7 was expressed at low levels, but E6 was not detected (Bohl et al., 2001). Analogous to the expression of $p12^{I}$ and $p30^{II}$ in HTLV-1-transformed T cell lines, neither E5 nor E7 proteins were detected in transformed C127 cells, even though the proteins were biologically active (Bohl et al., 2001). An important question that remains to be answered is whether $p12^{I}$ and $p30^{II}$ proteins and their mRNAs are expressed at higher levels in vivo compared to cell lines grown in vitro. It is possible that the proteins are naturally expressed at low levels but in sufficient amounts to accomplish their biological functions. Alternatively, the HTLV-1-transformed cell lines may present a snapshot of a particular aspect of the HTLV-1 gene expression program. In vivo, levels of pX mRNAs may be modulated by cellular factors in response to specific environmental stimuli and could be expressed at higher levels at specific points in the virus life cycle.

In the absence of an in vitro model system that recapitulates the in vivo HTLV-1 life cycle, we asked whether viral regulatory proteins or cellular splicing factors could modulate pX mRNA levels in transfection experiments. We were unable to detect significant shifts in specific pX mRNA levels in cells transfected with Tax-minus or Rex-minus proviruses, arguing against a role for Tax or Rex as repressors of pX mRNA expression. Overexpression of the splicing factors SF2/ASF and hnRNP A1 have been shown to modulate alternative RNA splicing in other retrovirus systems (Amendt et al., 1994; Bilodeau et al., 2001; Blanchette and Chabot, 1999; Chung and Derse, 2001; Damgaard et al., 2002; Gontarek and Derse, 1996; Jacquenet et al., 2001; Sun et al., 1993; Tange et al., 2001; Tange and Kjems, 2001). SF2/ASF belongs to the SR family of proteins involved in both constitutive and alternative splicing and whose activity is dependent on cell type, differentiation, or activation status (Fu, 1995). When cotransfected with HTLV-1 provirus, SF2/ASF caused a decrease in pX-tax-orf II and pX-rex-orf I mRNA levels relative to pX-tax/rex mRNA, suggesting that pX mRNA expression could be modulated in a condition-specific manner. The protein hnRNP A1 was previously shown to oppose the effects of SF2/ASF (Caceres et al., 1994). Although it did not affect the utilization of the alternative pX splice acceptor sites when overexpressed in transfected cells, it did cause exon 2 skipping, resulting in increased levels of pX-p21rex and pX-orfI mRNAs. These

cotransfection experiments provided a limited test of cellular factors that could potentially affect pX mRNA alternative splicing; further analyses of the viral *cis*-acting elements and the cellular *trans*-acting factors that interact to modulate pX mRNA and protein expression are in progress.

Materials and methods

Cells and transfections

The human T cell line HUT78 and HTLV-1-infected human T cells, MS9, MT2, and C8166, were grown in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. MS9 cell-culture medium also contained 100 U/ml of IL-2. Transformed human kidney 293T cells were maintained in DMEM with 10% fetal calf serum and antibiotics. Human 293T cells (3×10^6 cells in 10-cm plates) were transfected with 10 μg of plasmid DNA by calcium phosphate coprecipitation as previously described (Shuh et al., 1999).

Plasmids

The plasmid pCMV-HT1 contains the full-length HTLV-1 provirus in which the 5′ LTR was replaced by a CMV promoter containing a 5′ splice site (Shuh et al., 1999). pCMVHT-Tax9Q was derived from pCMV-HT1 by site-directed mutagenesis, which changed a CAG (Gln) codon in Tax to TAG (stop); this C-to-T change at position 6970 did not affect the overlapping Rex amino acid sequence. pCMVHT-Rex1L was also constructed by site-directed mutagenesis of pCMV-HT1 to change the Rex initiation codon from ATG (Met) to CTG (Leu). The A-to-C change is at nucleotide position 4772 of viral genomic mRNA. Mutations were confirmed by nucleotide sequencing and phenotypes were verified in functional assays. A portion of each alternatively spliced HTLV-1 mRNA, encompassing the characteristic splice junction, was amplified by RT-PCR from chronically infected cell lines and cloned into the pCRII-TOPO vector (Invitrogen). The cloned cDNAs were verified by nucleotide sequencing and were used to establish calibration curves in real-time PCR reactions or to define primer specificity. The following cDNA clones are listed by name with the virus sequence positions and exon definitions in parentheses; splice sites are indicated by slash marks. pB5-5 (78–119/4658–4831/6950–7278; ×1/×2/6950); pMS9-2.1 (4819–4831/6383–6551; ×2/6383); pMS9-11.1 (4819–4831/6478–6551; ×2/6478); pMS9-7.8 (78–119/6950–7278; ×1/6950); pMT2-2.3 (105–119/6383–6552; ×1/6383); pMT2-5.4 (105–119/6478–6552; ×1/6478); pMT2-2.8 (105–119/4658–4921; ×1/env4658); pMS9-8 (105–119/4641–4921; ×1/env4641).

RNA purification and cDNA synthesis

HTLV-I-infected cell lines, MS9, MT2, C8166, and transfected 293T cells, were harvested and washed with phosphate-buffered saline. Total RNA was extracted from the cells using the RNeasy kit (Qiagen) and poly(A^+)RNA was then prepared using the Oligotex kit (Qiagen). All quantitations were done by 260-nm absorbance measurements. cDNAs were then prepared by reverse transcription of 0.5 to 1.0 μg of poly(A^+) mRNA using Omniscript RT (Qiagen) as described by the manufacturer's protocol.

Real-time PCR

Real-time PCR and analysis of cDNA amplification products were performed on a Prism 7700 sequence detector (Perkin Elmer/Applied Biosystems, Foster City, CA), using 96-well optical plates and strip caps from the same supplier. The reaction conditions were 50°C for 2 min, 95°C for 10 min to activate the DNA polymerase, and then 50 cycles of 15 s at 94°C and 1 min at 60°C. All reaction volumes were 25 μl, including 5 μl of cDNA template, 20 μl of a PCR master mix (Applied Biosystems), 100 ng of each primer, and water. Calibration curves were derived by running 10-fold dilutions of the various cDNA plasmids diluted in TLE (20 mM Tris–HCl, pH 7.5; 0.1 mM EDTA) over the range of 2.5×10^0 to 2.5×10^7 copies. The cDNA samples were run at several dilutions. Each assay included duplicate wells for each dilution of calibration plasmids, triplicate wells for each sample cDNA, and controls which included nonspecific plasmid DNA, buffer, or water, all in duplicate. The threshold cycle values were used to plot the calibration curve. Standard curve data were graphed using Microsoft EXCEL 2000, which derived the equation for the line and calculated the coefficient of variation (CV). All standard curves had a CV of at least 0.97. The copy number of a particular cDNA in a sample was calculated from its measured Ct value with respect to the calibration plot. The copy numbers were normalized to the human GAPDH values measured in separate real-time PCR assays with the GAPDH kit (Perkin Elmer/Applied Biosystems) and GAPDH DNA standards from Intergen, Inc. All copy numbers derived are the result of at least six determinations. Real-time PCR reactions were performed using SYBR green or Taqman detection methods with nearly identical results.

PCR primers

The 5′ splice site-specific PCR primer sequences are summarized in Fig. 2. Other primers included: ex1/44641env, 5′-TCCGCCGTCTAGCTTC-3′; ex1/4658env, 5′-TCCGCCGTCTAGCCGC-3′; 3′-6552, 5′-GGAGAAAGCAGGAAGAGC-3′; 3′-7170, 5′-GAGTCGAGGGATAAGGAAC-3′; 3′-4921, 5′-CTGTAATCACCGAAGATGAG-3′; 3′-6552, 5′-GGAGAAAGCAGGAAGAGC-3′; and 3′-7170, 5′-GA-

GTCGAGGGATAAGGAAC-3′. For real-time PCR reactions in which a Taqman approach was used, the probe for ×1/6383, ×1/6478, ×1/×2/6383, or ×1/×2/6478 cDNAs was TMP-1 (P12/30TQM): 5′FAM-TTCGCCT-TCTCAGCCCCTTGTCT-TAMRA-3′. The Taqman probe for ×1/6950 and ×1/×2/6950 cDNAs was TMP-2 (HT039-TQM): 5′-FAM-ATCACCTGGGACCCCATC-TAMRA-3′.

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